Altered Neurotrophin Expression in a Mouse Model for Traumatic Brain Injury

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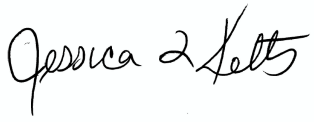

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ABSTRACT:

Neurotrophins are critical signaling molecules for cells of the central nervous system, as they have the ability to regulate proliferation, differentiation, and apoptosis. Neurotrophin expression can be influenced by sensory input, which has implications for both normal development of the central nervous system and remodeling that may occur in response to altered sensory input. We examined neurotrophin expression in the visual cortex in response to retinal degeneration as a model for Traumatic Brain Injury (TBI). We used a well-characterized mouse model system in which loss of function of a visual signaling molecule (phosphodiesterase 6β) causes retinal degeneration and loss of visual acuity in all mice by postnatal day (PND) 49. Using Q-PCR, we showed that expression of several neurotrophins, including BDNF, NT-3, and NGF was altered at the mRNA level in response to retinal degeneration. We also examined the protein expression levels of BDNF and NT-3 using western blotting. These analyses showed a decrease in BDNF protein levels at PND 49 in response to retinal degeneration, consistent with mRNA results; NT-3 protein levels did not reflect mRNA alterations that were seen in response to retinal degeneration, suggesting that NT-3 expression is regulated post-transcriptionally. The alterations in neurotrophin expression could have implications for remodeling in response to the loss of sensory input to the visual cortex, which can be related to TBI.

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INTRODUCTION:

TRAUMATIC BRAIN INJURY:

Traumatic Brain Injury (TBI) currently affects approximately 3.2 to 5.3 million people in the United Sates (Finan, 2018). The Centers for Disease Control (CDC) define TBI as “Injury to head arising from blunt or penetrating trauma or from acceleration – deceleration forces that are associated with symptoms or signs attributable to the injury” (Thurman, et al., 1999). Signs and symptoms of TBI include decreased levels of consciousness, amnesia, skull fractures, diagnosed intracranial lesions, neurological and neuropsychological abnormalities, or death (Thurman, et al., 1999; Roozenbeek, et al., 2013). From the millions of people affected, TBI claims 52,000 lives per year in the United States alone (Faul, et al., 2010). TBI is most commonly caused by events such as vehicle accidents, sports-related injuries, combat, physical abuse, or age-related accidents. There are several lasting medical issues surrounding TBI, including encephalopathy, Parkinson’s Disease, and diminished quality of life (Crane et al., 2016; Gardner et al., 2015). Overall, TBI causes a multitude of cognitive and physical interruptions that could possibly be reduced, if more thoroughly investigated. It is also important to note that the long-term effects of TBI have recently been examined and shown to have severely hazardous effects. Because TBI is such a prevalent issue, it is important to investigate ways in which the brain reacts to trauma.

NEUROTROPHINS:

Neurotrophins are protein growth factors that are involved in cell differentiation, proliferation, survival, and death. They are synthesized a proproteins, or pro-neurotrophins, that are cleaved to give rise to mature proteins. Four different neurotrophins have been well-characterized in mammals, including, Neurotrophin – 3 (NT-3), Neurotrophin – 4 (NT-4), Nerve Growth Factor (NGF), and Brain-Derived Neurotrophic Factor (BDNF). These neurotrophin proteins become especially important when brain injuries occur that cause a damaged network of brain cells. This is because the neurotrophins are involved in creating new neural connections and strengthening those that are already existing, as described below.

BDNF:

BDNF is linked to neuronal survival and function and is also highly expressed in hippocampal and cortical structures (Chao et al., 2006). BDNF is the neurotrophin has been found to be highly involved in synaptogenesis and neural connectivity, as well as the formation and maintenance of dendritic structure (Bing Hu and Cohen-Cory, 2005). Because of these roles involving brain plasticity, BDNF is of great interest in recovery from TBI. Mature BDNF has been shown to bind to a specific receptor called Tropomyosin receptor kinase B (TrkB) (Bramham and Messaoudi, 2005). When bound to its receptor, the BDNF-TrkB

complex will then serve as a docking site for second messengers aiding in the synaptic plasticity process (Bramham and Messaoudi, 2005). Research has also shown that these TrkB receptors are expressed in oligodendrocytes (Santi et al., 2006). When BDNF binds to TrkB in oligodendrocytes, proliferation was promoted, causing the brain remodeling (Santi et al., 2006).

NT-3:

NT-3 is found in the hippocampus, cerebellum, and spinal cord (Koyama et al., 2005). There are also other brain regions, known as sub-populations of neuronal cells, that constitutively produce NT-3 (Koyama et al., 2005). Like BDNF, NT-3 was determined to also be produced by astrocytes, which serves to convert resting astrocytes to reactive astrocytes (Mele et al., 2010, Koyama et al., 2005). This phenomenon poses an interest because astrocytes compose such a large percentage of brain cells, as well as play an important role in support of health and diseased brains. More information will be provided on astrocytes in later sections. Also, like BDNF, NT-3 has been linked to neuronal survival and function and is also highly expressed in cortical structures (Chao et al., 2006). NT-3 function has also been connected to the responsibilities of reduction of neuronal cell death, regeneration of damaged neurons, and promotion of regeneration of the spinal cord, septum, and striatum (Koyama et al., 2005).

NGF:

NGF is a secreted protein that can induce neurite outgrowth (Carleton et al., 2018). The classic targets for NGF include the sensory and sympathetic neurons of the PNS (Oshima and Guroff, 1996). However, NGF is a hormone/growth factor, it is found in almost all locations in the brain and PNS, to support the growth of nerves (Chao et al., 2006). NGF was determined to be responsible for the transport of nutrients to nerves, encourages neurite growth, produces cellular hypotrophy, and increases expression of enzymes involved in neurotransmitter synthesis (Oshima and Guroff, 1996). NGF also mediates other activities such as memory, behavior, and survival of neurons in the central nervous system (CNS) when processed into mature NGF (Chao et al., 2006). When NGF is processed into its mature form, it is highly involved in cell survival mechanisms activated by tyrosine kinase receptor A (TrkA).

NT-4:

NT-4 was the last of the neurotrophins to have been characterized. It can be located in several areas of the brain but is heavily localized in the hippocampus and amygdala regions. Like NGF, NT-4 was found to mediate activities such as memory, behavior, and survival of neurons in the central nervous system (CNS) when processed into mature NT-4 (Chao et al., 2006). NT-4 also enhanced glutamatergic synaptic transmission in cultured hippocampal neurons (Xie et al., 2000). Along with BDNF, mature NT-4 associates with the TrkB receptor. Studies have shown when the NT-4 gene was deleted, deficiencies in long-term memory

for contextual and auditory fear conditioning were observed (Xie et al., 2000). Fear conditioning is associated with the regions of the brain that strongly express NT-4, mainly the hippocampus. Another consequence of an NT-4 deficiency is a disruption of memory consolidation (Xie et al., 2000). NT-4 has been shown to be critically involved in activity-dependent, long-term synaptic plasticity (Xie et al., 2000).

PRO-NEUROTROPHINS AND PROPROTEIN CONVERTASES:

All four of the neurotrophins are synthesized in their pro-form and must be activated by endoproteolytic cleavage. Proprotein convertases (PCs) comprise a family of subtilisin-like, eukaryotic, serine endoproteases (Seidah and Chretien, 1999). These endoproteases work to activate a variety of proteins, including the aforementioned neurotrophins, growth factors, receptors, cell adhesion molecules, and metalloproteases (Bresnahan et al., 1990). Several PCs have been characterized, including furin, PACE4, PC5, PC7, and PC3 (Seidah and Chretien, 1999). PCs have also been shown to be expressed in several areas of the brain, allowing them a role in processing neurotrophins (Seidah et al., 1996). Each neurotrophin is cleaved by one or more of the PCs, either intracellularly, extracellularly, or both, to yield the mature neurotrophin. Significantly, when pro-neurotrophins were not cleaved by the PCs, they retained biological activity, but function quite differently than the mature forms. Pro-neurotrophins are proapoptotic and can affect long tern potentiation in the brain through interactions with the $p75^{NTR}$ receptor (Jarvinen et al., 2010). The function of pro-

neurotrophins versus that of mature forms are thus antagonistic, where pro-neurotrophins promote apoptosis and mature neurotrophins promote differentiation and proliferation. This makes the expression of both PCs and neurotrophins significant and examining how this expression changes in response to TBI could have considerable importance. PC expression has been shown to change in response to retinal degeneration, with potential implications for neurotrophin activity; for example, pro-BDNF is known to be upregulated during early postnatal brain development, potentially increasing the amount of mature BDNF after PC processing (Jarvinen et al., 2010). However, it is unknown how levels of pro-BDNF are affected by TBI or how changes in PC expression impact BDNF function in response to TBI, making investigation into these areas potentially significant.

Pro-NT3 is the precursor to mature NT-3. It has been established that pro-NT3 is primarily found in Müeller cells and photoreceptors in the brain (Shen et al., 2013). The amount of pro-NT3 available is directly related to the availability, or upregulation, of both mature NT-3 and the $p75^{NTR}$ receptor. The increase in pro-NT3 promotes degeneration of the healthy retina, which is part of photoreceptor homeostasis (Shen et al., 2013). Little is known about pro-NT3 and mature NT-3 levels before, during, and after trauma to the brain. For this reason, investigation into NT-3 is of great importance.

SIGNALING:

TBI, as well as the visual impairment process, cause significant brain remodeling to occur. Another way to describe brain remodeling is brain plasticity. Brain plasticity is the ability of different types of brain cells, like neurons and glial cells, to create new connections to promote brain remodeling. When a TBI occurs, brain remodeling is a key factor in regenerating healthy neural connections, and therefore, healthy brain activities. To promote brain plasticity, neurotrophins are released. The neurotrophins are unique for many reasons, one being they exert cellular effects through two different receptors, Trk (tyrosine kinase family receptors) and, as mentioned before, $p75^{NTR}$ (Chao et al., 2006). When mature neurotrophins bind to the Trk receptors, they activate them, which in turn regulates cell proliferation and survival, axonal and dendritic growth and remodeling, assembly of the cytoskeleton, membrane trafficking and fusion, and synapse formation and function (Bramham and Messaoudi, 2005). Pro-neurotrophins will bind to the $p75^{NTR}$ receptor to promote cell death pathways (Chao et al., 2006). As presented in Figure 1, once the neurotrophins are processed by the PCs, the mature neurotrophin will bind to their appropriate Trk receptor and promote cell proliferation and differentiation. Or, if they are not processed by PCs, the pro-neurotrophin will binds to the $p75^{NTR}$ receptor and promote apoptosis.

Figure 1: Proprotein Convertase Processing of Neurotrophins

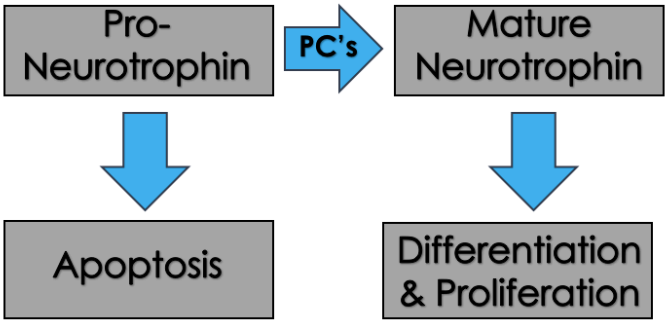

Figure 1: Flow chart of the proprotein convertase (PC) processing of pro-neurotrophins to give rise to mature neurotrophins, therefore increasing cell differentiation and proliferation. Pro-neurotrophins signal cell death.

The neurotrophin-Trk complex will then serve as a docking site for second messengers aiding in the synaptic plasticity process (Bramham and Messaoudi, 2005). Research has also shown that these Trk receptors are expressed in oligodendrocytes (Santi et al., 2006). When BDNF binds to TrkB in oligodendrocytes proliferation is promoted, causing the brain remodeling (Santi et al., 2006). NGF binds as a complex to a Trk receptor, specifically the TrkA receptor. This cell cell-survival is depicted in Figure 2 (Chao et al., 2006).

Figure 2: Trk Receptor Binding

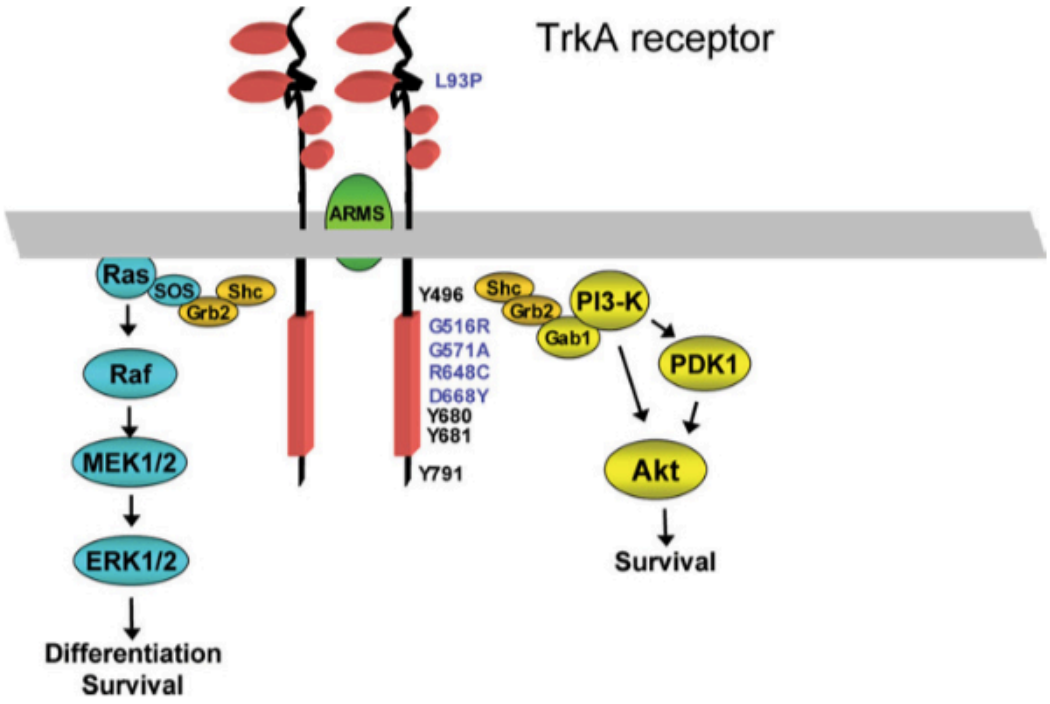

Figure 2: Trk receptor structure and the consequences of neurotrophin binding. Resultant signaling promotes cell differentiation, proliferation, and survival (Chao et al., 2006).

Once the mature neurotrophins bind to the Trk receptor, the activated Trk receptors recruit adaptor proteins and effectors. At this point the adaptor proteins, Shc and FRS2, influence signal transduction and cross-talk, while the effectors, PI3K and PLC-γ, are molecules that selectively bind to proteins to regulate biological activity. The two main pathways activated by neurotrophin binding to the Trk receptors are the PI3/Akt and Ras/Erk pathways (Chao et al.,2006). Both of these are well-characterized pathways that promote cell differentiation and survival. In contrast to mature neurotrophin binding to Trk

receptors, when pro-neurotrophins bind to the $p75^{NTR}$, apoptosis is induced. In Figure 3 we see the pathways in which apoptosis is induced.

Figure 3: $p75^{NTR}$ Receptor Binding

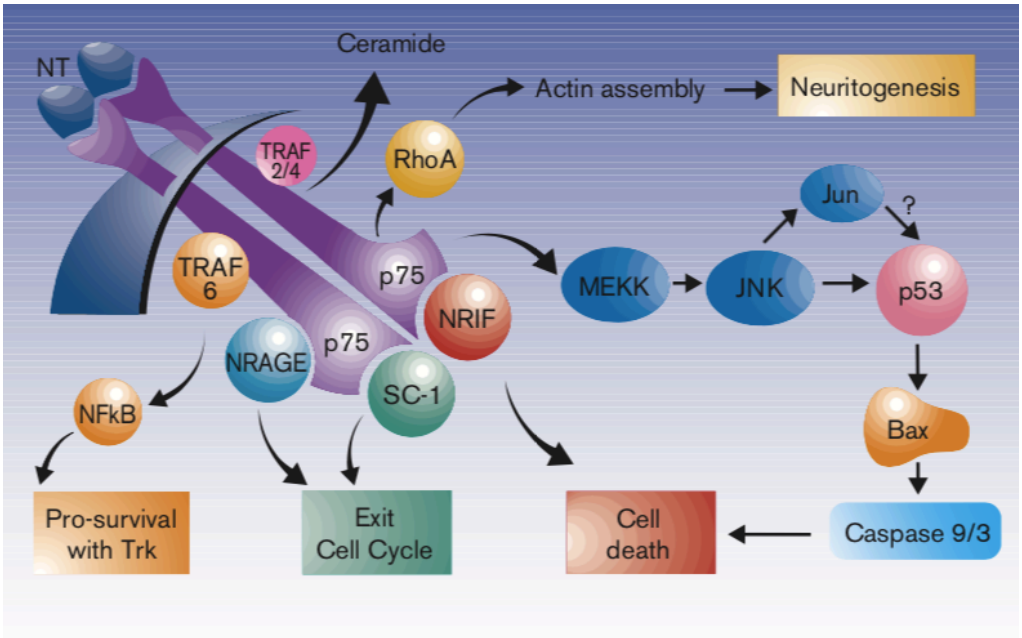

Figure 3: Pro-neurotrophin binding to $p75^{NTR}$ receptor induces apoptotic pathways. (Kaplan and Miller, 2000)

The binding of pro-neurotrophins to the $p75^{NTR}$ receptor engages several proteins and pathways. Most proteins involved in this process induce cell death or an exit from the cell cycle causing eventual cell senescence. These apoptotic pathways are induced by proteins SC-1, NRIF1, NRAGE. This will cause an increase in the level of cellular ceramide causing the JNK-p53-Bax pathway to activate and cause cell death (Kaplan and Miller, 2000).

TBI MOUSE MODEL:

We have utilized a mouse model to investigate TBI for this study. According to a study by Finan called “Biomechanical Stimulation of Traumatic Brain Injury in the Rat,” a major reason for choosing a mouse model instead of another animal model, such as a rat, is because there is still a gap in TBI experimental data for producing traumatic brain injuries in the lab (Finan, 2018). To test the effects of TBI in a lab setting, visual impairment was selected to use as a model for brain trauma, rather than other methods that have proved crueler and give less consistent results. Mice with a nonsense mutation in their phosphodiesterase-6 beta (Pde6β) gene will lose all sight by postnatal day (PND) 49. This is a naturally occurring mutation in the Pde6β subunit gene, which is expressed in rod photoreceptors and causes a disease called retinitis pigmentosa (Hart, el al., 2005; Cornett et al., 2011). A functional Pde6β gene contributes to a heterotetrametric phosphodiesterase complex that regulates cytoplasmic cGMP levels in rod photoreceptors in response to light stimuli (Hart, el al., 2005). When the Pde6β subunit is mutated, there will be an elevated cGMP level because a permanent opening of the cGMP-gated cation channel (Figure 4) in the membrane of the rod photoreceptors (Hart, el al., 2005). This continuous opening in the cGMP-gates cation channel leading to apoptosis, causing blindness to occur (Hart, el al., 2005). Phenotyping has determined that this mutation produces a slow progression of retinal degeneration, causing total blindness in mice by PND 49, which is beneficial for lab studies (Hart, el al., 2005). Because the Pde6β- mutation destroys rod photoreceptors, the brain must react. This

reaction is known as remodeling. In a study performed by Cornett et al. (2011), Pde6β-, or retinal degeneration (RD), mice demonstrated glial plasticity based on mRNA data in the RD mice at PND 28 and 49, as well as significant changes in astrocyte densities (Cornett et al. 2011). Due to the occurrence of brain remodeling with the Pde6β- mutation, like that of TBI, it makes the RD mouse model a good fit for research on TBI. Figure 4 is a pictorial representation of what occurs in the rod photoreceptors in RD mice.

Figure 4: Pictorial Representation of the Pde6β- Mutation

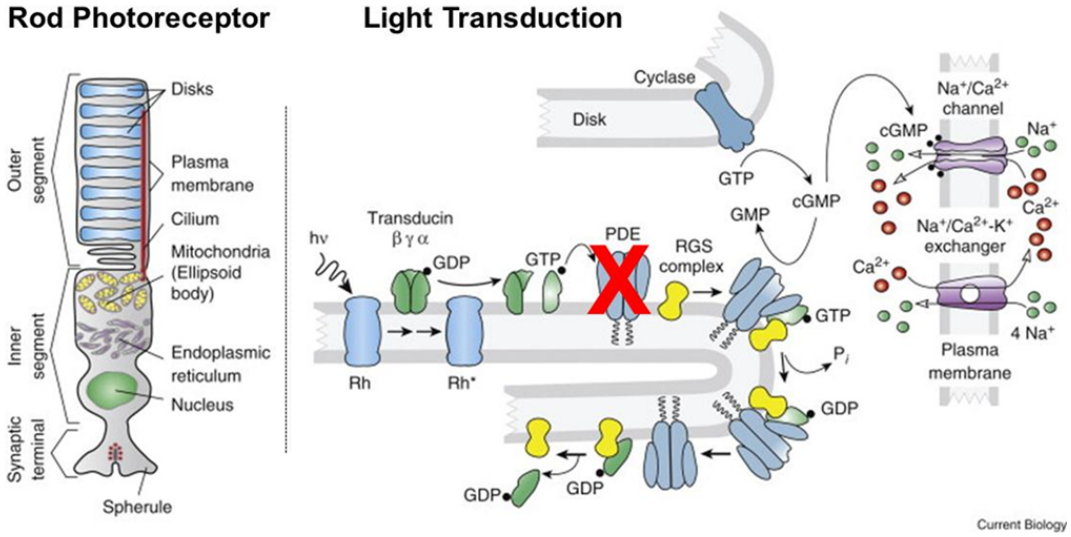

Figure 4: An explanation of the mechanism of the Pde6β- mutation. The left side of Figure 4 shows the layout of the rod photoreceptor. The right side of Figure 4 is a detailed look into the mutation of the Pde6β- subunit of a rod photoreceptor. The mutation is labeled with a red "X", which leaves the ion channel open, leading to increased cytoplasmic cGMP and eventual apoptosis (Fain, et al., 2010.

BRAIN REGIONS AND CELL TYPES OF INTEREST:

The complex nature of the brain has researchers looking into the many regions it possesses. For this project, the focus is on part of the cerebral cortex called the visual cortex (VC). The VC is the part of the cerebral cortex that receives and processes sensory nerve impulses from the eyes. Mammalian visual systems establish visual pathways based on input received from the retina (Cornett et al., 2011). These stimuli establish the correct visual pathways for sight (Cornett et al., 2011). In order to continue to shape the neural connections in the VC, a combination of spontaneous stimuli-evoked activities must perpetually occur (Cornett et al., 2011). In the developing mouse, normal neural circuitry is established by PND 35 (Cornett et al., 2011).

The VC is comprised of different types of glial cells. Glial cells include several different types of neural cells including satellite cells, Schwann cells, oligodendrocytes, astrocytes, ependymoglia, and Müeller and Bergman glial cells. Glial cells, such as astrocytes and oligodendrocytes, are supportive cells to both the central nervous system (CNS) and peripheral nervous system (PNS). These support cells carry nutrients and provide protection to other neural cells, including neurons. Oligodendrocytes are known as the myelinating cells in the CNS (Michalski and Kothary, 2015). The myelination process is crucial to neuron survival and activity; without myelination, neurons cannot maintain ion gradients, and will therefore affect impulse ability and strength (Michalski and Kothary, 2015). BDNF is found in many areas of the brain and is implicated in brain

plasticity and remodeling, as stated previously. Synaptic activity between neurons, including that from TBI, induces activity-dependent release of BDNF in cells (Kuczweski et al., 2009). This is important because BDNF is widely abundant in the hippocampus, amygdala, and cerebellum, and is mainly expressed in neurons, astrocytes, and is secreted by dendrites, and therefore promotes synaptic regeneration and survival (Kuczweski et al., 2009). One type of glial cell, astrocytes, are a type of specialized cell that outnumber neurons by over fivefold (Sofroniew and Vinters, 2010). Astrocytes are the most abundant population of glial cells, making up nearly half of human brain cells, and one-third of mice brains (Ge et al., 2012; Cahoy et al., 2008). These cells are critical to the regulation of synaptic connectivity and strength (Cahoy et al., 2008). Astrocytes function to mediate transport of waste products and nutrients between blood vessels and neurons, as well as modulate neuronal activity and synaptic transmission (Reuss, 2009). In the VC, astrocytes comprise approximately 28% of all cells and 60% of glial cells (Cornett et al., 2011). Early in post-natal development, astrocytes reach adult-like numbers and have roles in synaptogenesis, neural plasticity, and modification of neurotransmitter release (Cornett et al., 2011). Astrocytes are responsible for supporting oligodendrocytes by secreting BDNF and NT-3 once there are signs of white matter ischemic injury (Miyamoto et al., 2015). This means that BDNF and NT-3 promote proliferation and differentiation of oligodendrocyte precursor cells into lineage cells (Miyamoto et al., 2015). The neurotrophins from these astrocytes also help to restore myelin function in neurons (Miyamoto et al., 2015). For these reasons, levels of pro- and

mature neurotrophins can vary throughout astrocytes, oligodendrocytes, and neurons depending on the trafficking of these proteins and the availability of proprotein convertases (PCs), such as furin, as well as the level of TBI or visual impairment in this study. Glial cells are a major source of neurotrophins in healthy and distressed central and peripheral nervous systems (Reuss, 2009). Neurotrophins have an important role in the regulation of cellular repair processes after injury, degeneration, and demyelination in both the CNS and PNS (Reuss, 2009).

Preliminary data indicated mature BDNF expression is decreased in RD mice, compared to normal wild-type mice. Western blotting was performed to determine the expression levels of BDNF in both WT and RD mice. Those tests were performed using the visual cortices of numerous mice. This preliminary examination established the groundwork for future work with neurotrophin production. Based on this information, it is hypothesized that pro-neurotrophin levels will increase in the damaged areas of the brain because of their role in apoptosis, while mature neurotrophins could increase in other brain areas to promote brain remodeling. Therefore, for this study, the following was examined:

SPECIFIC AIMS:

AIM 1: Determine mRNA levels of BDNF, NT-3, NT-4, and NGF from VC of mice throughout the process of normal vision to visual impairment.

AIM 2: Determine levels of pro-BDNF and mature BDNF from VC of mice throughout the process of normal vision to visual impairment.

AIM 3: Determine levels of pro-NT3 and mature NT-3 in VC of mice throughout the process of normal vision to visual impairment.

The Pde6β- mutation that causes visual impairment serves as a model for TBI, which is testable for differences in the levels of neurotrophins. Mice were observed throughout postnatal days 14, 28, and 49 to view changes in neurotrophin levels as visual impairment sets in due to the Pde6β- mutation. In this study NT-4, NGF, BDNF, and NT-3 at the mRNA level and NT-3 and BDNF at the protein level will be the main areas of interest.

METHODS AND MATERIALS:

ANIMALS:

All mice were housed individually in standard cages in colony rooms with a 12:12 (LD) cycle, with food and water provided *ad libitum*. Animals were bred for the purpose of this study, and all protocols were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan and Emmanuel College. Separate groups of mice were used for each phase of the experiment.

This study utilized a commercially available (Jackson Laboratories; Stock #003257) transgenic mouse strain, FVB/N-Tg(GFAP-GFP-Mes/J (Zhuo et al., 1997), hereafter called RD mice. The RD mouse has a naturally-occurring nonsense mutation in exon 7 of the phosphodiesterase 6 β subunit (Pde6β) gene (Pittler and Baehr, 1991) expressed in rod photoreceptors (Bowes, et al., 1990). This causes cGMP levels to accumulate (Farber and Lolley, 1974) and leads to a moderate rate of retinal degeneration that occurs in several well-defined stages (Marc et al., 2003).

An appropriate behavioral control for the RD mice is the congenic mouse strain, FVB.129P2-Pde6β+Tyr$^{c\text{-}ch}$/AntJ, hereafter called WT mice (Jackson Laboratories; Stock #004828). This strain originated on a FVB;129P2 background and is homozygous for the 129P2/OlaHsd wildtype Pde6β allele. Backcrossed for least

11 generations on the FV background, WT mice do not show any evidence of retinal degeneration.

VERIFICATION OF PDE6β- MUTATION:

To begin, the difference between the WT mice and the RD mice had to be established. A simple test was used to verify the Pde6β- mutation. At PND 21, 28, 35, 42, and 49, the Pde6β- and WT mice were lowered onto sand paper of varying grade (smooth, medium, and coarse). Mice were held by the tail and lowered down to a surface of sandpaper, where they would splay legs (or not) before impact. WT mice would splay legs while by PND 49 RD mice would not, therefore demonstrating visual impairment.

RNA EXTRACTION AND cDNA SYNTHESIS:

Tissue from the visual cortices was removed from normal and RD mice at different developmental time points (post-natal days 14, 28, and 49). Total RNA was extracted from the tissue using the PureLink RNA Mini kit (Life Technologies; Carlsbad, CA). The Superscript III kit (Life Technologies) was used as a template for cDNA synthesis. The cDNA was used for quantitative real-time PCR as described below.

QUANTITATIVE REAL-TIME PCR:

Primers specific for the amplification of sequences coding for mouse BDNF, NT-3, NGF, NT-4, and mouse glyceraldehyde-3-phosphate dehydrogenase

(GAPDH; used as a reference gene) were obtained from Qiagen (Valencia, CA). Quantitative Real-Time PCR was done using the RT^2 SYBR Green Mastermix (Qiagen). Reaction volumes and cycling conditions were set up according to the instructions provided by Qiagen. Amplification was done using a Mastercycler ep $Realplex^4$ thermal cycler (Eppendorf; Westbury, NY). All samples were done in triplicate. Ct values were generated for each reaction and melting curve analysis was done to ensure only one product was amplified in each reaction. Changes in gene expression were analyzed by the ΔΔCt method using the two closest Ct values from each set of triplicates, as long as they were within one amplification cycle. These values were averaged to generate ΔCt values, which were in turn used to generate the ΔΔCt as described previously (Cornett et al. 2011). Figure 5 is an outline of the Q-PCR tissue preparation, protocol, and data analysis.

Figure 5: Outline of Real Time PCR Protocol

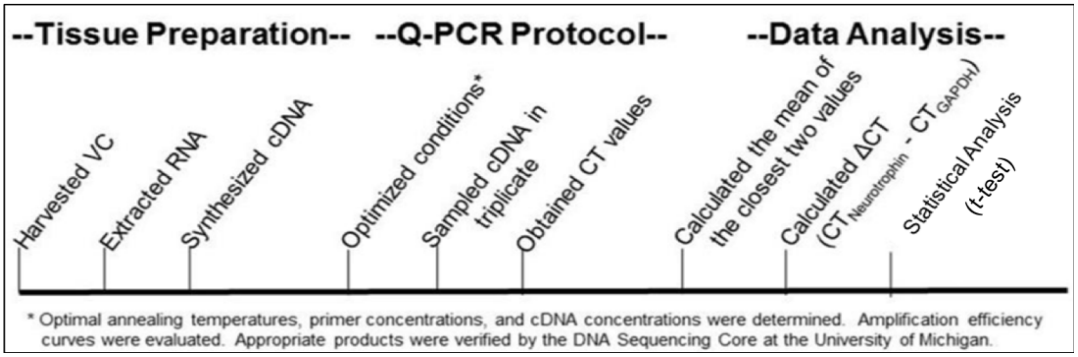

Figure 5: Outline of the real time PCR analysis.

WESTERN BLOTTING:

Brain tissue was removed from VC from WT and RD mice (n = 48) at different development times (postnatal days 14, 28, and 49). Tissue was vigorously triturated in ice-cold lysis buffer (50 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate), centrifuged at 10,000 g for 10 min to remove debris, and the supernatant was stored at -80° C. Protein concentrations in the lysates were determined using a Pierce® BCA Protein Assay kit (Thermo Fisher; Waltham, MA) and 10 ug (BDNF/NT-3 analysis) and 12-23 ug (NT-3 analysis) total protein were subjected to SDS-PAGE (Bolt 4-12% gradient resolving gel; Life Technologies). Resolved proteins were transferred to Immobilon-P membranes (Millipore; Billerica, MA). The Immobilon was blocked for 1 hr (room temperature, on an orbital shaker set to 50 rpm) in TBS-Tween (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) containing 3% non-fat milk. Polyclonal rabbit antibodies to BDNF (Abcam; Cambridge, MA), NT3 (Thermo Fisher), and β-actin (Thermo Fisher) were used for the blotting. Antibodies were diluted into the blocking solution (1:500 for anti-BDNF, 1:1000 for anti-NT3, and 1:2000 for anti-β-actin) and incubated overnight at room temperature on an orbital shaker (50 rpm). The blot was then washed three times (3 minutes each) with TBS-Tween (20 ml each wash) and blocked again for 30 minutes. Anti-rabbit IgG conjugated to alkaline phosphatase (Promega; Madison, WI) was diluted 1:2500 into the blocking solution and incubated for 1.5 hr at room temperature (50 rpm on orbital shaker). The blot was washed twice (3 minutes each) with TBS-Tween (20ml each wash), and once (3

min) with western blot substrate buffer (0.1 M Tris-HCl [pH 9.5], 0.1 M NaCl, 5mM $MgCl_2$).

DATA & STATISTICAL ANALYSIS:

SPSS for Windows (Version 16) was used for analysis of mRNA expression using a t-test with age as the between-subject variable interpreting any significant factor interactions and for all tests, $p<0.05$ was considered statistically significant (Jarvinen et al., 2010). The analysis was used to determine differences in gene expression from RD and WT brain lysates.

Western Blot analysis was performed using Image J software from the National Institute of Health (NIH). To perform Image J analysis, we opened the image of the blot in Image J and, using the triangle tool, we drew a border around each band and selected analyze. This was carried out for each band on each western blot. The straight-line tool was used to connect the base of each peak and then to remove background between each band. Each peak was then highlighted, and the area of the peak appeared in a separate window. An analysis of the percent saturation peaks was then obtained. This information was copied and pasted into a Microsoft Excel document. These data were normalized against a β-actin band and then t-tests were performed for statistical analysis.

RESULTS:

To begin, the difference between WT mice RD mice was established as described previously in the methods section. Figure 6 shows the results of this test. By PND 49, the RD mice showed no evidence of normal visual activity.

Figure 6: Verification of Retinal Degeneration in Pde6β- Transgenic Mice

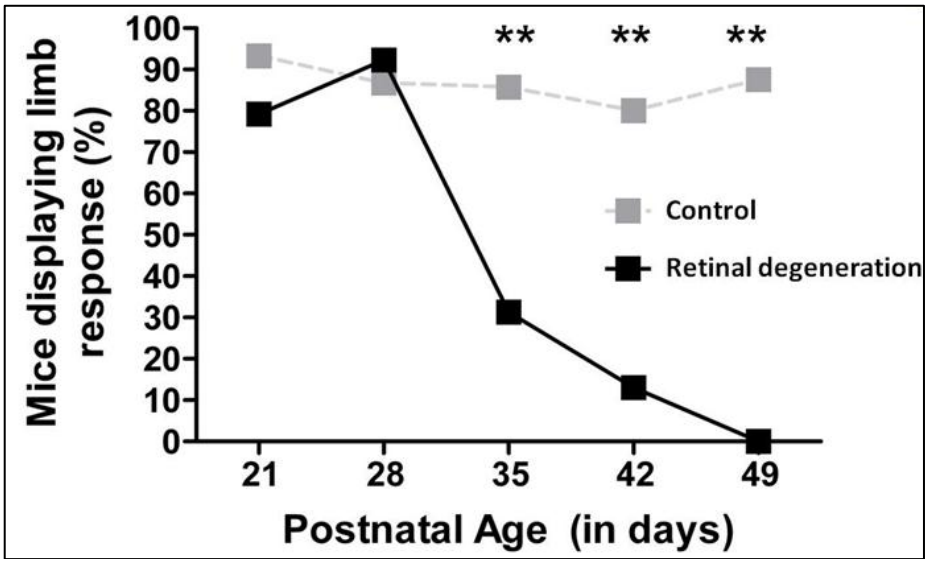

Figure 6: Verification of retinal degeneration in RD mice. To determine loss of vision at days 21, 28, 35, 42, and 49, the RD and WT mice were lowered onto sand paper of varying grade (smooth, medium, and coarse). Mice were held by the tail and lowered down to a surface of sandpaper, where they would splay legs (or not) before impact. Mice with normal visual activity would splay legs while mice with defective vision would not. ** Significance was determined by t-test, $p < 0.05$.

To examine molecular events that might be driving remodeling, we looked at mRNA expression of the four neurotrophins in the VC before (PND 14), during (PND 28), and after (PND 49) the onset of blindness. In Figure 7 we see the

analysis of BDNF mRNA in RD and WT mice. In WT mice, BDNF mRNA levels steadily increased between PND 14 and 49, with a very steep increase between PND 28 and 49. This increase was not seen in RD mice, which actually show a decrease in BDNF mRNA at PND 49. There was a significantly higher ($p < 0.05$) level of BDNF mRNA observed in the VC of WT mice at PND 49.

Figure 7: Expression of BDNF mRNA in RD Mice

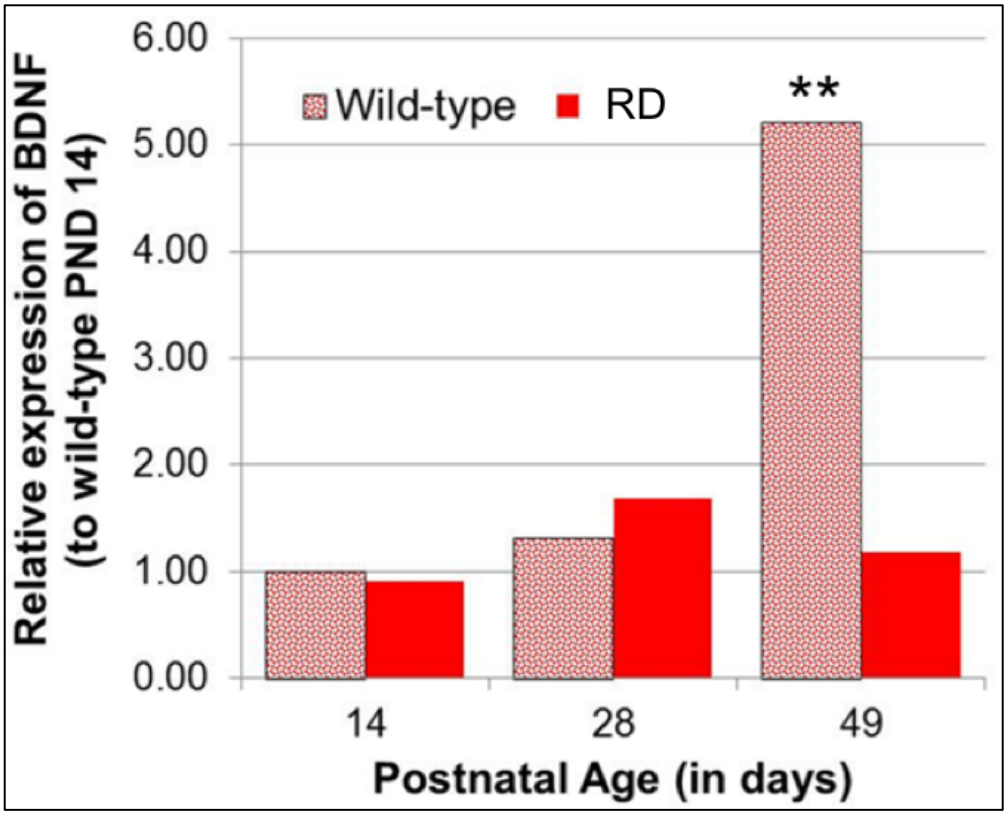

Figure 7: Analysis of BDNF mRNA expression in wild type and retinal degeneration mice at postnatal days 14, 28, and 49. Changes in gene expression were analyzed by the ΔΔCt method using the two closest Ct values from each set of triplicates, as long as they were within one amplification cycle. These values were averaged to generate ΔCt values, which were in turn used to generate the ΔΔCt. Expression in RD was normalized to WT BDNF expression at PND 14. **Significance was determined by t-test, $p < 0.05$.

Figure 8 shows that in WT mice, NT-3 mRNA levels decreased between PND 14 and 49, but this decrease was not seen in RD mice, who showed steady or increasing NT-3 mRNA expression. There was a significantly higher ($p < 0.05$) level of NT-3 mRNA observed in the VC of RD mice at PND 49.

Figure 8: Expression of NT-3 mRNA in RD Mice

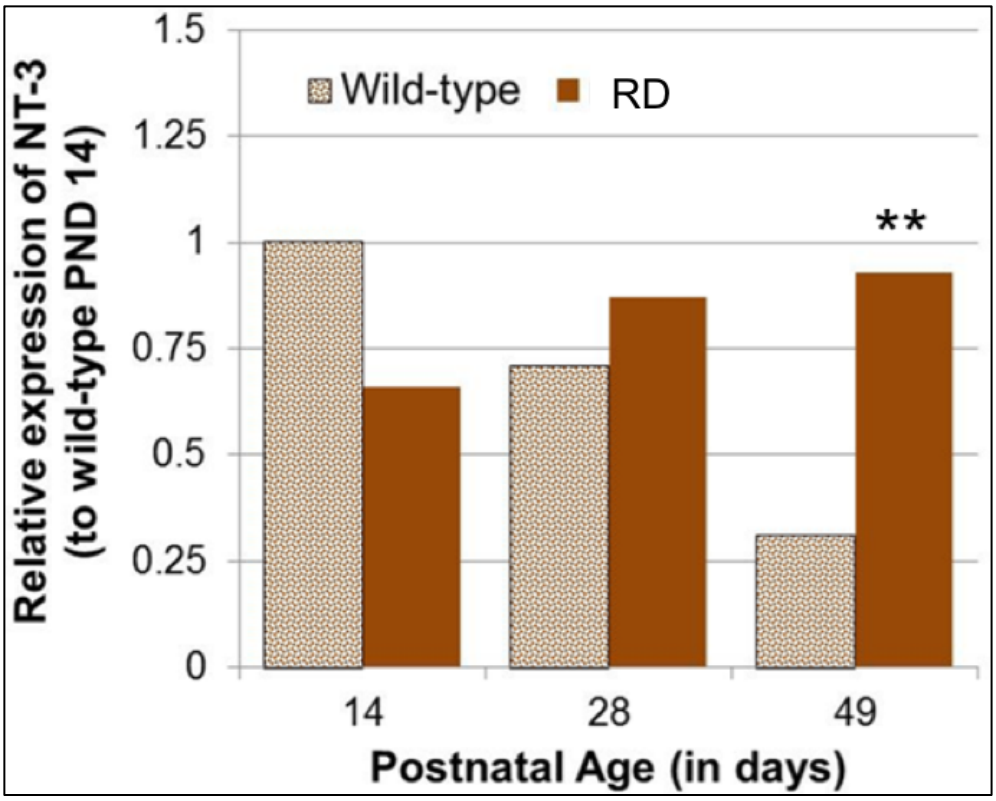

Figure 8: Analysis of NT-3 mRNA expression in wild type and retinal degeneration mice at postnatal days 14, 28, and 49. Changes in gene expression were analyzed by the ΔΔCt method using the two closest Ct values from each set of triplicates, as long as they were within one amplification cycle. These values were averaged to generate ΔCt values, which were in turn used to generate the ΔΔCt. Expression in RD was normalized to WT NT-3 expression at PND 14. **Significance was determined by t-test, $p < 0.05$.

Figure 9 shows the analysis of NGF mRNA in RD and WT mice. In WT mice, NGF mRNA levels steadily decreased between PND 14 and 49. In RD mice, this trend is delayed. In RD mice, the decrease does not occur until after PND 28, resulting in a significant ($p < 0.05$) increase in RD vs. WT NGF mRNA at PND 28. This is an increase in NGF mRNA expression in Pde6β- mice compared to WT NGF mRNA expression.

Figure 9: Expression of NGF mRNA in RD Mice

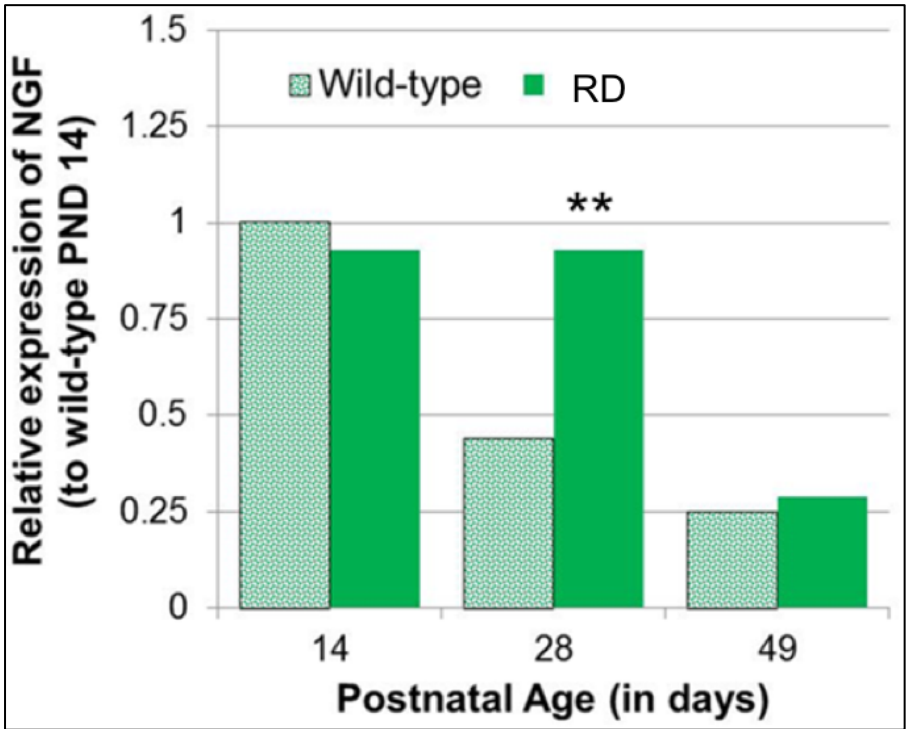

Figure 9: Analysis of NGF mRNA expression in wild type and retinal degeneration mice at postnatal days 14, 28, and 49. Changes in gene expression were analyzed by the ΔΔCt method using the two closest Ct values from each set of triplicates, as long as they were within one amplification cycle. These values were averaged to generate ΔCt values, which were in turn used to generate the ΔΔCt. Expression in RD mice was normalized to WT NGF expression at PND 14. **Significance was determined by t-test, $p < 0.05$.

Figure 10 shows the analysis of NT-4 mRNA in Pde6β- and WT mice. In both WT and Pde6β- mice, there is a steady increase in NT-4 mRNA expression from PND 14 to 49. This resulted in no significant ($p < 0.05$) differences in RD vs. WT NT-4 mRNA.

Figure 10: Expression of NT-4 mRNA in RD Mice

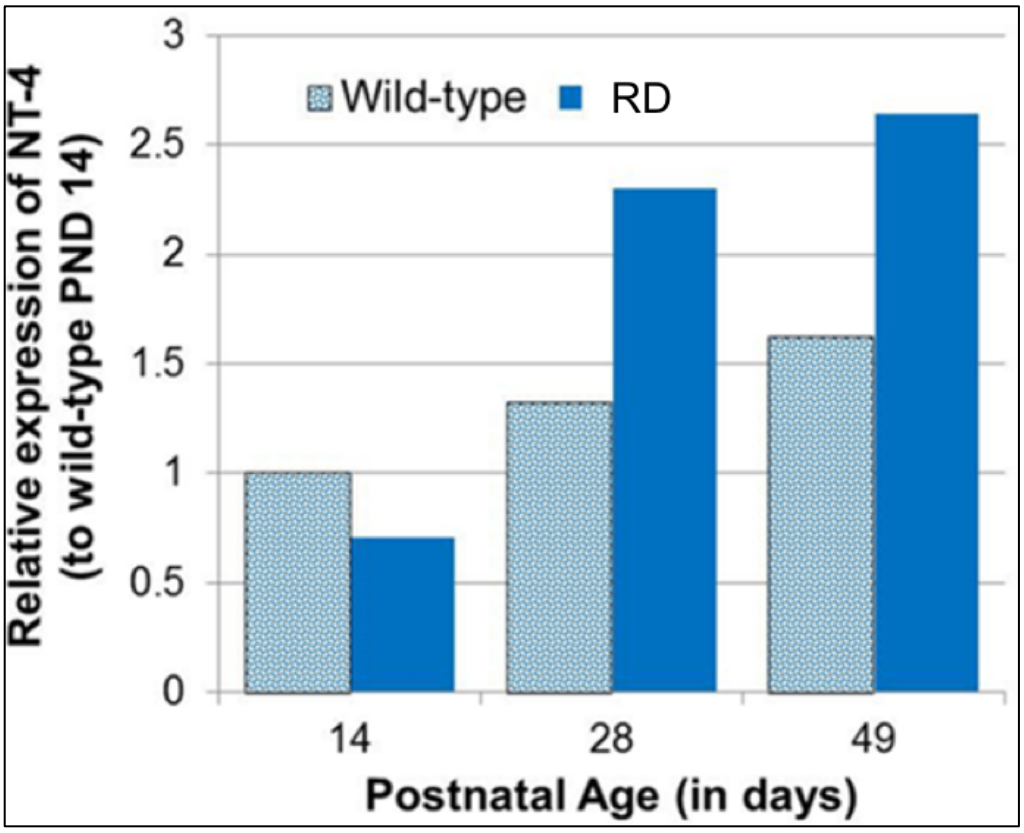

Figure 10: Analysis of NT-4 mRNA expression in wild type and retinal degeneration mice at postnatal days 14, 28, and 49. Changes in gene expression were analyzed by the ΔΔCt method using the two closest Ct values from each set of triplicates, as long as they were within one amplification cycle. These values were averaged to generate ΔCt values, which were in turn used to generate the ΔΔCt. Expression in RD was normalized to WT NT-4 expression at PND 14. **Significance was determined by t-test, $p < 0.05$.

Figure 11 shows the combined Q-PCR results for the four neurotrophins and highlights significant differences in expression that were found at PND 28 for NGF, and PND 49 for NT-3 and BDNF. Significance is marked by ** where $p < 0.05$.

Figure 11: mRNA Expression of Neurotrophins in RD Mice

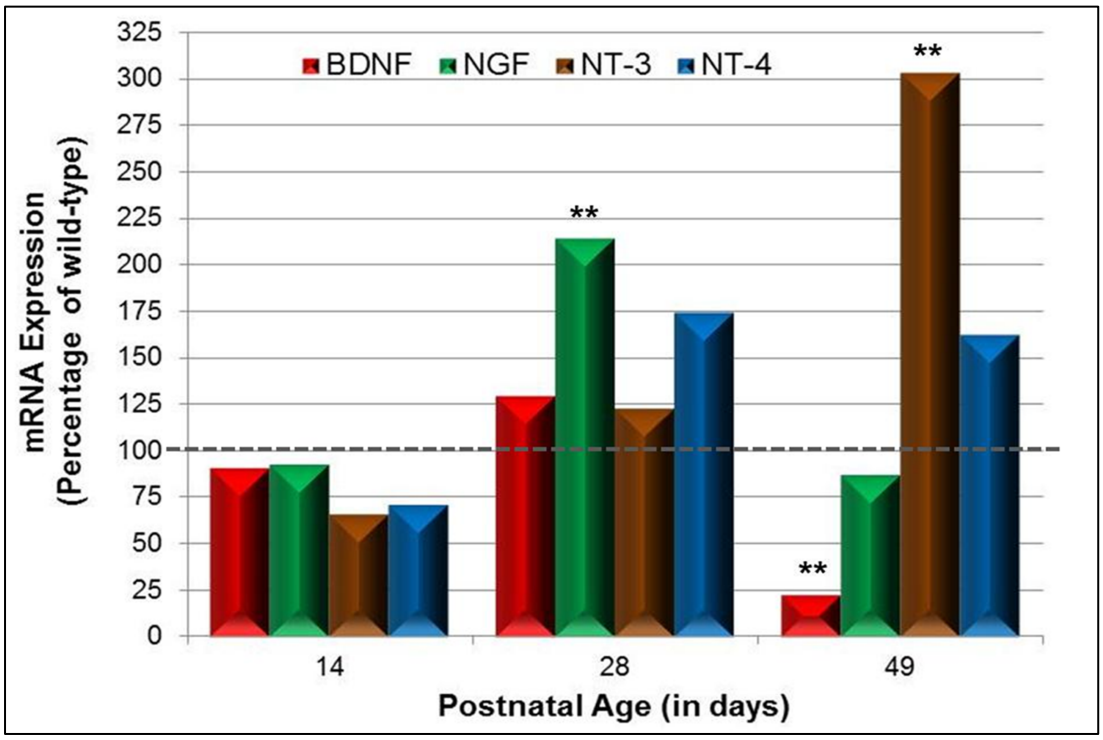

Figure 11: Q-PCR results for BDNF, NGF, NT-3, and NT-4. Graph displays mRNA expression for neurotrophins BDNF, NGF, NT-3, and NT-4 at PND 14, 29, and 49 in RD mice. Changes in gene expression were analyzed by the ΔΔCt method using the two closest Ct values from each set of triplicates, as long as they were within one amplification cycle. These values were averaged to generate ΔCt values, which were in turn used to generate the ΔΔCt. Expression was normalized to WT at PND 14. **Significance was determined by t-test, $p < 0.05$.

We next decided to investigate if neurotrophin protein levels matched the changes in mRNA levels that were seen for BDNF and NT-3. We used western blotting to detect the proteins, quantified the results with Image J, and normalized the results to the β-actin control. Because the pro- and mature forms of these neurotrophins can elicit dramatically different biological efforts, we analyzed the levels of both their pro- and mature forms.

Figure 12 shows the combined results of all BDNF western blot data. The RD mice showed decreased levels of both pro- and mature forms of BDNF at both PND 14 and 49. The decrease seen at PND 49 is consistent with mRNA analysis, in which the RD mice had a much lower expression level at PND 49.

Figure 13 shows the combined results of all NT-3 western blot data. No differences were observed in pro-NT-3 levels at any postnatal age. However, at PND 14, the RD mice showed a higher level of mature NT-3.

Figure 12: Combined Western Blot Data for pro- and Mature BDNF

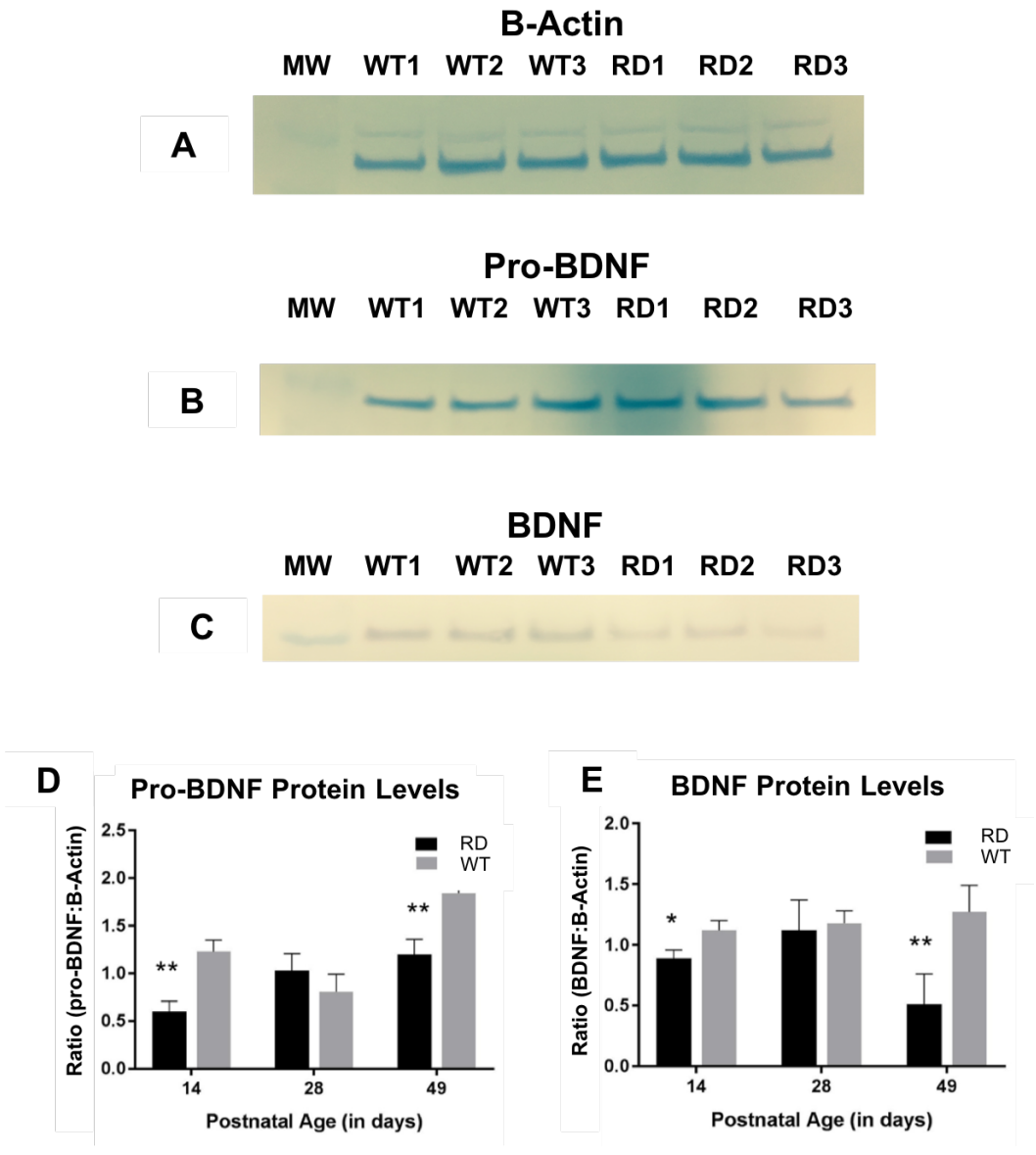

Figure 12: Combined western blot data for pro- and mature BDNF. Panel A shows a B-actin sample western blot containing 3 WT samples and 3 RD samples. Panel B shows a pro-BDNF sample western blot containing 3 WT samples and 3 RD samples. Panel C shows a BDNF sample western blot containing 3 WT samples and 3 RD samples. Panel D illustrates all pro-BDNF results at PND 14, 28, and 49 in WT and RD mice. Panel E is a graph of all mature BDNF results at PND 14, 28, and 49 in WT and RD mice. **Significance $p < 0.05$.

Figure 13: Combined Western Blot Data for pro- and Mature NT-3

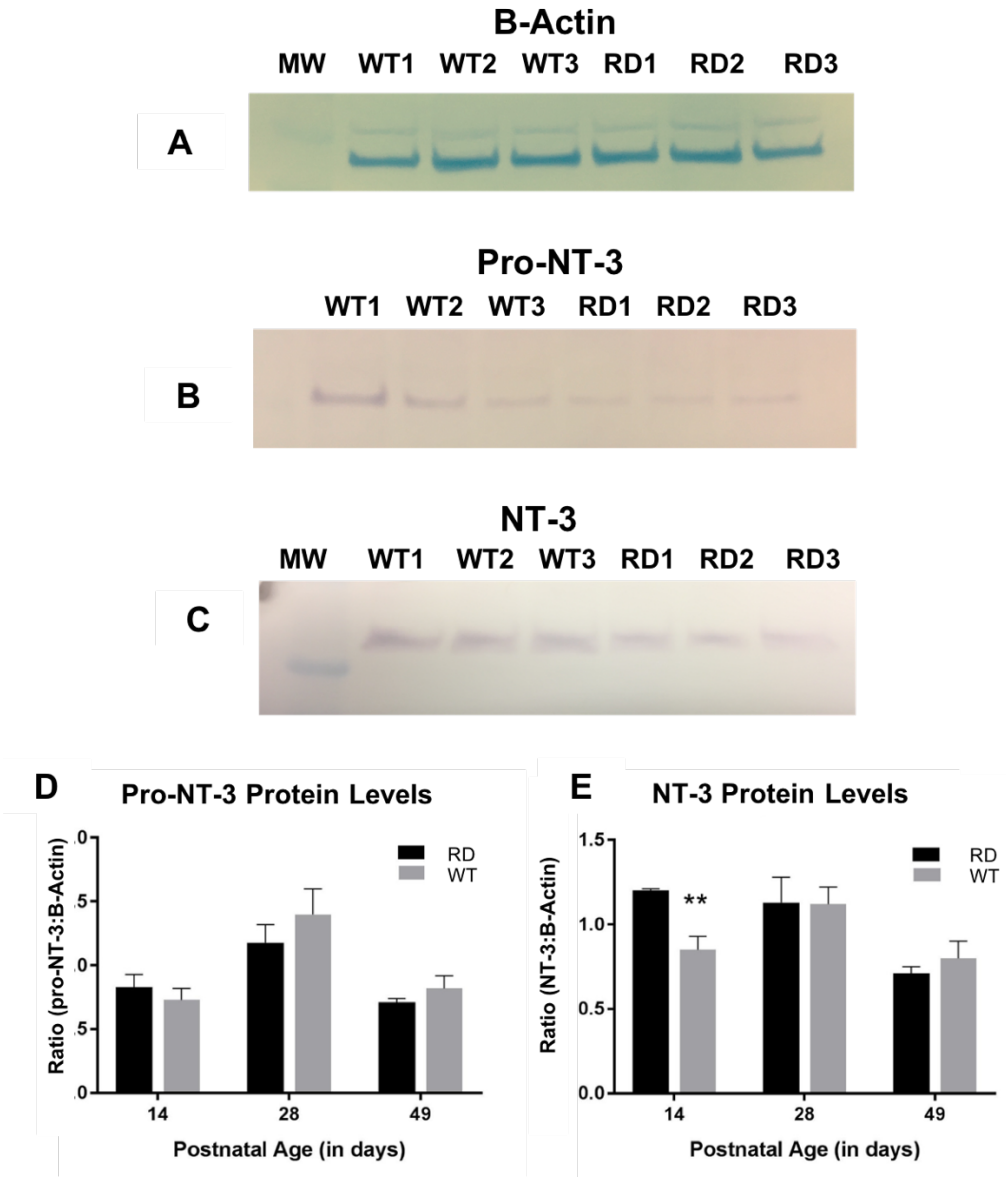

Figure 13: Combined western blot data for pro- and mature NT-3. Panel A shows a B-actin sample western blot containing 3 WT samples and 3 RD samples. Panel B shows a pro-NT-3 sample western blot containing 3 WT samples and 3 RD samples. Panel C shows a mature NT-3 sample western blot containing 3 WT samples and 3 RD samples. Panel D illustrates all pro-NT-3 results at PND 14, 28, and 49 in WT and RD mice. Panel E is a graph of all mature NT-3 results at PND 14, 28, and 49 in WT and RD mice. **Significance $p < 0.05$.

DISCUSSION:

The goal of this study was to investigate neurotrophin levels in the brain under traumatic circumstances. The first step to accomplish this was to identify a model of TBI. RD mice homozygous for a loss of function mutation in the Pde6β gene could provide a good model as they lose vision in early postnatal development. Vision loss was verified through a simple test, wherein at days 21, 28, 35, 42, and 49, the RD and WT mice were lowered onto sand paper of varying grade (smooth, medium, and coarse) where they would splay legs (or not) before impact. WT mice would splay legs while mutant mice would not. Once verification of vision loss was completed, we investigated expression of neurotrophins whose production and processing could be critical to events in TBI. It is known that protein production can be regulated at several points during gene expression. To determine whether neurotrophin production is regulated at the transcription, translation, or post-translational level we examined mRNA and protein levels for these proteins in RD and WT mice at various PND time points. Quantitative real-time PCR (Q-PCR) was utilized to analyze the mRNA levels of BDNF, NT-3, NT-4, and NGF in VC tissue samples. Changes in gene expression were analyzed by the ΔΔCt method using the two closest Ct values from each set of triplicates, as long as they were within one amplification cycle. These values were averaged to generate ΔCt values, which were in turn used to generate the ΔΔCt as described previously (Jarvinen et al., 2010). Following the mRNA analysis using Q-PCR, western blotting was to examine protein expression. The protein level of both pro- and mature neurotrophins, BDNF and NT-3 was examined.

Protein expression does not always directly correlate with the amount of mRNA present, which is why both levels (mRNA and protein) were assessed.

VISUAL IMPAIRMENT ALTERS BDNF LEVELS

Because neurotrophins are implicated in cell differentiation, proliferation, survival, and cell death, an overarching hypothesis that in times of traumatic brain injury, the level of mature neurotrophins would increase, leading to proliferation. Specifically within the VC in this study, it could also be hypothesized that an increase in pro-neurotrophins could be seen, leading to cell death, as well as a decrease in mature neurotrophins because of the damage occurring to the VC during vision loss. Specifically, for BDNF, the latter was seen. BDNF mRNA levels steadily increased in WT mice between PND 14 and 49, with a very steep increase between PND 28 and 49. This increase was not seen in RD mice, which actually show a decrease in BDNF mRNA at PND 49. There was a significantly higher ($p < 0.05$) level of BDNF mRNA observed in the VC of WT mice at PND 49 compared to the RD mice. Alongside the mRNA data, the protein analysis confirms a decrease in both pro- and mature BDNF levels in RD mice during the process of retinal degeneration. This could reflect the role of mature BDNF in establishing neural connectivity (Bung Hu and Cohen-Cory, 2005). In the visual cortex of RD mice, there is no need for new neural connections, because the vision has been lost by PND 49. The overall lower amount of pro- and mature BDNF at both PND 14 and PND 49 in RD mice could be significant as sensory deprivation could be starting by PND 14 (Jarvinen et al., 2010; Cornett et al.,

2011). In WT mice, the difference in BDNF protein levels at PND 14 to PND 49 could be explained by the developing brain; more neural connections would be established during development as compared to PND 49 in the RD mice when all vision has been lost.

NT-3 LEVELS ARE AFFECTED BY VISUAL IMPAIRMENT

Investigation into NT-3 expression revealed interesting and contradictory results. First, Q-PCR results showed that NT-3 mRNA levels remained high during PND 14-49, in contrast to WT levels, which decreased. These results were contradicted by the protein data produced by western blot analyses. In Figure 13D, no significant differences were seen in pro-NT3 levels between RD and WT mice at any postnatal time period (Figure 13E). However, at PND 14, there is a significant increase in mature NT-3 in RD mice compared to WT mice. A potential explanation for this could be due to the levels of PCs available for processing proproteins to mature proteins in RD mice during this timeframe. The PCs, PCSK9 and furin, have both been shown to increase at the level of mRNA during postnatal weeks 1-3 in RD mice (Jarvinen et al., 2010). Because NT-3 is expressed in the VC during development and visual impairment, and based on the level of the aforementioned PCs, the amount of mature NT-3 could go up at PND 14 compared to WT (Jarvinen et al., 2010; Cornett et al., 2011). This could make sense considering the role that mature NT-3 plays in neuronal survival (Chao et al., 2006). With sensory deprivation beginning around PND 14, production of mature NT-3 could be induced to counteract, at least short-term,

neuronal death. Another possible reason for the discrepancy between the NT-3 mRNA and protein levels could be due to an increased stress response causing an increase in mRNA, but for the same reason, a stress response, causing the translation into protein being stopped for conservation of resources in the dying VC. While there is currently no documentation of neurotrophin expression being regulated at the level of translation, this is a commonly used regulatory mechanism. A well-studied example of regulation of at the translational level is the transferrin receptor. This regulation is dictated by several things. For example, the level of cellular stress, amount of available iron, and regulatory proteins all dictate translation of the transferrin receptor mRNA (Theil, 1990). Like the transferrin receptor, NT-3 could also be regulated at this stage.

FUTURE EXPERIMENTATION:

Future experimentation of protein analysis for NGF would provide a more comprehensive analysis of the neurotrophins. This could be done like the BDNF and NT-3 analysis utilizing western blotting and Image J. The key time point for this analysis could be PND 28, where RD mice show higher NGF mRNA levels than WT mice (Figure 9).

Further experimentation utilizing fluorescence-activated cell sorting (FACS) could provide insight into levels of neurotrophin mRNA and protein levels in specific cell types. The mice selected for this study have fluorescently tagged astrocytes, neurons, and oligodendrocytes. Once separated by FACS, the samples can be

processed like all other samples for this project and subjected Q-PCR and Western blotting. This information would provide vital knowledge, because each cell-type has different functions that require different growth factors. This testing could give specific results showing which neurotrophins are most pertinent in specific cell types. Identifying specific neurotrophin information for specific cell types may provide future therapeutic options targeting neurotrophins.

REFERENCES:

Bing Hu, A., Nikolakopoulou, M., Cohen-Cory, S. (2005). BDNF stabilizes synapses and maintains the structural complexity of optic axons in vivo. *Development, 132*(19), 4285-4298.

Bramham, C. R., Messaoudi, E. (2005). BDNF function in adult synaptic plasticity: The synaptic consolidation hypothesis. *Progress in Neurobiology, 76*(2), 99-125.

Bresnahan, P. A., Richard Leduc, Laurel Thomas, Thorner, J., Gibson, H.L., Brake, A. J., Thomas, G., (1990). Human fur gene encodes a yeast KEX2-like endoprotease that cleaves pro-β-NGF in vivo. *The Journal of Cell Biology, 111*(6), 2851-2859.

Cahoy, J. D., Emery, B., Kaushal, A., Foo, L. C., Zamanian, J. L., Christopherson, K. S., Barres, B. A. (2008). A transcriptome database for astrocytes, neurons, and oligodendrocytes: A new resource for understanding brain development and function. *Journal of Neuroscience, 28*(1), 264-278.

Carleton, L. A., Chakravarthy, R., Van Der Sloot, A. M., Mnich, K., Serrano, L., Samali, A., Gorman, A. M. (2018). Generation of rationally-designed nerve growth factor (NGF) variants with receptor specificity. *Biochemical and Biophysical Research Communications, 495*(1), 700-705.

Chao, M. V., Rajagopal, R., & Lee, F. S. (2006). Neurotrophin signaling in health and disease. *Clinical Science (London, England: 1979), 110*(2), 167.

Crane, P.K., Gibbons, L.E., Dams-O'Connor, K., Trittschuh, E., Leverenz, J.B., Keene, C.D., Sonnen, J., Montine, T.J., Bennett, D.A., Leurgans, S., Schneider, J.A., Larson, E.B., (2016). Association of traumatic brain injury with late-life neurodegenerative conditions and neuropathologic findings. JAMA Neurol. 73, 1062–1069.

Fain, G., Hardie, R., Laughlin, S. B., (2010). Phototransduction and the Evolution of Photoreceptors. Current Biology 20(3). 1-23.

Farber, D., Lolley, R., (1974). Cyclic guanosine monophosphate: elevation in degenerating photoreceptor of cells of the C3H mouse retina. Science 186, 449-451.

Faul, M.X.L., Wald, M.M., Coronado, V., (2010). Traumatic Brain Injury in the United States: Emergency Department Visits, Hospitalizations, and Deaths, 2002–2006. CDC, National Center for Injury Prevention and Control, Atlanta, GA.

Finan, J. D. (2018). Biomechanical simulation of traumatic brain injury in the rat. *Clinical Biomechanics.*

Gardner, R.C., Burke, J.F., Nettiksimmons, J., Goldman, S., Tanner, C.M., Yaffe, K., (2015). Traumatic brain injury in later life increases risk for Parkinson disease. Ann. Neurol. 77, 987–995.

Hart, A. W., McKie, L., Morgan, J. E., Gautier, P., West, K., Jackson, I. J., & Cross, S. H. (2005). Genotype-phenotype correlation of mouse Pde6b mutations. *Investigative Ophthalmology & Visual Science, 46*(9), 3443-3450.

Jarvinen, M. K., Chinnaswamy, K., Sturtevant, A., Hatley, N., Sucic, J. F., (2010) Effects of age and retinal degeneration on the expression of proprotein convertases in the visual cortex. *Brain Research*. 1317. 1-12.

Kaplan, D. R., Miller, F. D. (2000). *Neurotrophin signal transduction in the nervous system*. England: Elsevier Ltd. 381-390.

Koyama, Y., Baba, A., Matsuda, T. (2005). Endothelins stimulate the expression of neurotrophin-3 in rat brain and rat cultured astrocytes. *Neuroscience, 136*(2), 425-433.

Marc, R.E., Jones, B.W., Watt, C.B., Strettoi, E., (2003). Neural remodeling in retinal degeneration. Prog. Retin. Eye Rec. 22, 607-655.

McKee, A.C., Stein, T.D., Kiernan, P.T., Alvarez, V.E., (2015). The neuropathology of chronic traumatic encephalopathy. Brain Pathol. 25, 350–364.

Mele, Tina, Carman-Krzan, Marija, Mojca Juric, Damijana. (2008). Regulatory role of monoamine neurotransmitters in astrocyte NT-3 synthesis. *International Journal of Developmental Neuroscience*. 28. 13-19.

Michalski, J.P., Kothary, R. (2015). Oligodendrocytes in a nutshell. *Frontiers in Cellular Neuroscience*. 9 (340). 1-11.

Miyamoto, N., Maki, T., Shindo, A., Liang, A. C., Maeda, M., Egawa, N., Arai, K. (2015). Astrocytes promote oligodendrogenesis after white matter damage via brain-derived neurotrophic factor. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 35*(41), 14002.

Oshima, M., Hirata, Y., & Guroff, G. (1996). The nerve growth factor family. *Growth factors and cytokines in health and disease* (pp. 229-258).

Pittler, S.J., Baehr, W., 1991. Identification of a nonsense mutation in the rod photoreceptor cGMP phosphodiesterase beta-subunit gene of the rd mouse. Proc. Natl. Acad. Sci. W.S.A. 88, 8322-8326.

Reuss, B. (2009). Glial growth factors. *819-825.*

Roozenbeek, B., Maas, A.I., Menon, D.K., 2013. Changing patterns in the epidemiology of traumatic brain injury. Nat. Rev. Neurol. 9, 231–236.

Santi, S., Schenk, U., Matteoli, M., Aicardi, G., Canossa, M., Bergami, M., Riccio, M. (2006). Hippocampal neurons recycle BDNF for activity-dependent secretion and LTP maintenance. *The EMBO Journal, 25*(18), 4372-4380.

Seidah, N. G., Chrétien, M. (1999). Proprotein and prohormone convertases: A family of subtilases generating diverse bioactive polypeptides. *Brain Research, 848*(1-2), 45-62.

Seidah, N. G., Benjannet, S., Pareek, S., Chrétien, M., Murphy, R. A. (1996). Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. *FEBS Letters, 379*(3), 247-250.

Shen, W., Zhu, L., Lee, S., Chung, S. H., & Gillies, M. C. (2013). Involvement of NT3 and P75(NTR) in photoreceptor degeneration following selective müller cell ablation. *Journal of Neuroinflammation, 10*, 137.

Sofroniew, M., & Vinters, H. (2010). Astrocytes: Biology and pathology. *Acta Neuropathologica, 119*(1), 7-35.

Xie, Cui-Wei, Sayah, David, Chen, Qi-Sheng, Wei, Wei-Zheng, Smith, Desmond, Liu, Xin. (2000) Deficient Long-Term Memory and long-lasting long-term potentiation in mice with a targeted deletion of neurotrophin-4 gene. *Proc. Natl. Acad. Sci. USA.* 97(14). 8116-8121.

Zhuo, L., Sun, B., Zhang, C.L., Fine, A., Chiu, S.Y., Messing A., (1997). Live astrocytes visualized by green fluorescent protein in transgenic mice. Dev. Biol. 187, 36-42.

APENDICES:

1) PND 14 PROTEIN ANALYSIS T-TEST

T-Test

Notes

Output Created		01-OCT-2017 20:09:08
Comments		
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	Weight	<none>
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	Cases Used	Statistics for each analysis are based on the cases with no missing or out-of-range data for any variable in the analysis.
Syntax		T-TEST GROUPS=Genotype(1 2) /MISSING=ANALYSIS /VARIABLES=BActin BDNF proBDNF NT3 proNT3 /CRITERIA=CI(.95).
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Group Statistics

	Genotype	N	Mean	Std. Deviation	Std. Error Mean
BActin	Wild-type	3	1.0350	.03730	.02153
	rd	3	1.0323	.09437	.05448
BDNF	Wild-type	3	1.1224	.13411	.07743
	rd	3	.8944	.11462	.06618
proBDNF	Wild-type	3	1.2275	.19964	.11526
	rd	3	.5964	.18367	.10604
NT3	Wild-type	3	.8549	.13103	.07565
	rd	3	1.1960	.01642	.00948
proNT3	Wild-type	3	.8299	.16486	.09518
	rd	3	.7286	.15319	.08845

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
BActin	Equal variances assumed	1.356	.309	.046	4
	Equal variances not assumed			.046	2.610
BDNF	Equal variances assumed	.121	.745	2.239	4
	Equal variances not assumed			2.239	3.905
proBDNF	Equal variances assumed	.112	.755	4.030	4
	Equal variances not assumed			4.030	3.972
NT3	Equal variances assumed	6.822	.059	-4.474	4
	Equal variances not assumed			-4.474	2.063
proNT3	Equal variances assumed	.005	.948	.780	4
	Equal variances not assumed			.780	3.979

Independent Samples Test

t-test for Equality of Means

		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower
BActin	Equal variances assumed	.966	.00269	.05858	-.15997
	Equal variances not assumed	.967	.00269	.05858	-.20055
BDNF	Equal variances assumed	.089	.22801	.10186	-.05479
	Equal variances not assumed	.090	.22801	.10186	-.05752
proBDNF	Equal variances assumed	.016	.63113	.15662	.19628
	Equal variances not assumed	.016	.63113	.15662	.19509
NT3	Equal variances assumed	.011	-.34112	.07624	-.55281
	Equal variances not assumed	.044	-.34112	.07624	-.65979
proNT3	Equal variances assumed	.479	.10139	.12993	-.25936
	Equal variances not assumed	.479	.10139	.12993	-.26013

Independent Samples Test

		t-test for Equality of Means 95% Confidence Interval of the Difference Upper
BActin	Equal variances assumed	.16534
	Equal variances not assumed	.20592
BDNF	Equal variances assumed	.51081
	Equal variances not assumed	.51354
proBDNF	Equal variances assumed	1.06599
	Equal variances not assumed	1.06718
NT3	Equal variances assumed	-.12943
	Equal variances not assumed	-.02245
proNT3	Equal variances assumed	.46214
	Equal variances not assumed	.46290

2) PND 28 PROTEIN ANALYSIS T-TEST:

T-Test

Notes

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	N of Rows in Working Data File	6
Missing Value Handling	Definition of Missing	User defined missing values are treated as missing.
	Cases Used	Statistics for each analysis are based on the cases with no missing or out-of-range data for any variable in the analysis.
Syntax		T-TEST GROUPS=Genotype(1 2) /MISSING=ANALYSIS /VARIABLES=BActin BDNF proBDNF NT3 proNT3 /CRITERIA=CI(.95).
Resources	Processor Time	00:00:00.00
	Elapsed Time	00:00:00.01

Group Statistics

	Genotype	N	Mean	Std. Deviation	Std. Error Mean
BActin	Wild-type	3	1.0799	.06924	.03998
	RD	3	1.1994	.20318	.11731
BDNF	Wild-type	3	1.1818	.17336	.10009
	RD	3	1.1195	.43342	.25024
proBDNF	Wild-type	3	.8050	.31480	.18175
	RD	3	1.0295	.31168	.17995
NT3	Wild-type	3	1.1217	.16826	.09715
	RD	3	1.1270	.25532	.14741
proNT3	Wild-type	3	1.4012	.34772	.20076
	RD	3	1.1800	.24363	.14066

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
BActin	Equal variances assumed	1.915	.239	-.964	4
	Equal variances not assumed			-.964	2.458
BDNF	Equal variances assumed	2.827	.168	.231	4
	Equal variances not assumed			.231	2.624
proBDNF	Equal variances assumed	.019	.898	-.878	4
	Equal variances not assumed			-.878	4.000
NT3	Equal variances assumed	.318	.603	-.030	4
	Equal variances not assumed			-.030	3.462
proNT3	Equal variances assumed	.942	.387	.902	4
	Equal variances not assumed			.902	3.582

3)

Independent Samples Test

		t-test for Equality of Means			
		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower
BActin	Equal variances assumed	.390	-.11949	.12393	-.46358
	Equal variances not assumed	.420	-.11949	.12393	-.56793
BDNF	Equal variances assumed	.829	.06224	.26951	-.68604
	Equal variances not assumed	.834	.06224	.26951	-.86937
proBDNF	Equal variances assumed	.429	-.22457	.25576	-.93468
	Equal variances not assumed	.430	-.22457	.25576	-.93471
NT3	Equal variances assumed	.978	-.00527	.17654	-.49543
	Equal variances not assumed	.978	-.00527	.17654	-.52702
proNT3	Equal variances assumed	.418	.22113	.24513	-.45946
	Equal variances not assumed	.424	.22113	.24513	-.49193

Independent Samples Test

		t-test for Equality of Means 95% Confidence Interval of the Difference Upper
BActin	Equal variances assumed	.22460
	Equal variances not assumed	.32894
BDNF	Equal variances assumed	.81052
	Equal variances not assumed	.99385
proBDNF	Equal variances assumed	.48554
	Equal variances not assumed	.48557
NT3	Equal variances assumed	.48489
	Equal variances not assumed	.51647
proNT3	Equal variances assumed	.90173
	Equal variances not assumed	.93419

3) PND 49 PROTEIN ANALYSIS T-TEST:

t-test

Notes

Output Created		01-OCT-2017 20:12:34
Comments		
Input	Data	D:\Laptop Backup 0621 2017\Research\nsf data\Stats analysis\Jarvinen PND 49 neurotrophin protein raw data 10012017.sav
	Active Dataset	DataSet1
	Filter	<none>
	Weight	<none>
	Split File	<none>
	N of Rows in Working Data File	7
Missing Value Handling	Definition of Missing	User defined missing values are treated as missing.
	Cases Used	Statistics for each analysis are based on the cases with no missing or out-of-range data for any variable in the analysis.
Syntax		T-TEST GROUPS=Genotype(1 2) /MISSING=ANALYSIS /VARIABLES=BActin BDNF proBDNF NT3 proNT3 /CRITERIA=CI(.95).
Resources	Processor Time	00:00:00.00
	Elapsed Time	00:00:00.01

[DataSet1] D:\Laptop Backup 0621 2017\Research\nsf data\Stats analysis\Jarvinen PND 49 neurotrophin protein raw data 10012017.sav

Group Statistics

	Genotype	N	Mean	Std. Deviation	Std. Error Mean
BActin	Wild-type	3	.9126	.10083	.05822
	RD	3	1.1299	.11834	.06832
BDNF	Wild-type	3	1.2723	.37975	.21925
	RD	3	.5147	.44092	.25457
proBDNF	Wild-type	3	1.8351	.25619	.14791
	RD	3	1.2003	.27694	.15989
NT3	Wild-type	3	.7984	.17707	.10223
	RD	3	.7122	.06870	.03966
proNT3	Wild-type	3	.8210	.16845	.09725
	RD	3	.7054	.04761	.02749

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
BActin	Equal variances assumed	.081	.790	-2.421	4
	Equal variances not assumed			-2.421	3.902
BDNF	Equal variances assumed	.055	.826	2.255	4
	Equal variances not assumed			2.255	3.914
proBDNF	Equal variances assumed	.068	.807	2.914	4
	Equal variances not assumed			2.914	3.976
NT3	Equal variances assumed	4.102	.113	.786	4
	Equal variances not assumed			.786	2.589
proNT3	Equal variances assumed	2.821	.168	1.144	4
	Equal variances not assumed			1.144	2.318

Independent Samples Test

t-test for Equality of Means

		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower
BActin	Equal variances assumed	.073	-.21729	.08976	-.46651
	Equal variances not assumed	.074	-.21729	.08976	-.46900
BDNF	Equal variances assumed	.087	.75764	.33597	-.17516
	Equal variances not assumed	.089	.75764	.33597	-.18330
proBDNF	Equal variances assumed	.043	.63477	.21781	.03002
	Equal variances not assumed	.044	.63477	.21781	.02858
NT3	Equal variances assumed	.476	.08617	.10965	-.21827
	Equal variances not assumed	.497	.08617	.10965	-.29635
proNT3	Equal variances assumed	.316	.11561	.10106	-.16499
	Equal variances not assumed	.357	.11561	.10106	-.26685

Independent Samples Test

		t-test for Equality of Means 95% Confidence Interval of the Difference Upper
BActin	Equal variances assumed	.03193
	Equal variances not assumed	.03443
BDNF	Equal variances assumed	1.69044
	Equal variances not assumed	1.69858
proBDNF	Equal variances assumed	1.23952
	Equal variances not assumed	1.24097
NT3	Equal variances assumed	.39062
	Equal variances not assumed	.46870
proNT3	Equal variances assumed	.39620
	Equal variances not assumed	.49806